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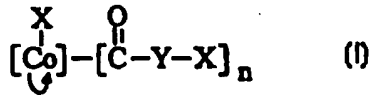
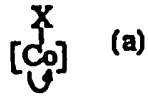
(54) Title: RADIONUCLIDE LABELING OF VITAMIN B_{12} AND COENZYMES THEREOF

(57) Abstract

A compound useful for *in vivo* imaging of organs and tumors is provided of formula (1) wherein (a) is cobalamin, (b) derived from a corrin carboxylic acid group of said cobalamin, Y is a linking group and X is a chelating group, optionally comprising a detectable radionuclide or a paramagnetic metal ion, and n is 1-3.



(I)



(a)

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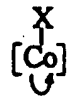
RADIONUCLIDE LABELING OF VITAMIN B_{12} AND COENZYMES THEREOF

Background of the Invention

For several years after the isolation of vitamin B_{12} as cyanocobalamin in 1948, it was assumed that cyanocobalamin and possibly hydroxocobalamin, its photolytic breakdown product, occurred in man. Since then it has been recognized that cyanocobalamin is an artifact of the isolation of vitamin B_{12} and that hydroxocobalamin and the two coenzyme forms, methylcobalamin and adenosylcobalamin, are the naturally occurring forms of the vitamin.

The structure of these various forms is shown in Figure 1, wherein X is CN, OH, CH_3 or adenosyl, respectively. Hereinafter, the term cobalamin will be used to refer to all of the molecule except the X group. The fundamental ring system without cobalt (Co) or side chains is called *corrin* and the octadehydrocorrin is called *corrole*. The Co-contg heptacarboxylic acid resulting from hydrolysis of all the amide groups without the CN and the nucleotide, is designated *cobyrinic acid*. The corresponding hexacarboxylic acid with D-1-amino-2-propanol side chain f is called *cobinic acid* and the hexacarboxylic acid with the α-D-ribofuranose-3-phosphate attached to the 2-position of the amino propanol is called *cobamic acid*. Thus, *cobamide* is the hexaamide of cobamic acid, *cobyric acid* is the hexaamide of cobyrinic acid and *cobinamide* is the hexaamide of cobinic acid. Figure 1 is adapted from The Merck Index, Merck & Co. (11th ed. 1989), wherein X is above the plane defined by the corrin ring and nucleotide is below the plane of the ring. The corrin ring has attached six amidoalkyl ($H_2NC(O)Alk$) substituents, at the 2, 3, 7, 8, 13, and 18 positions, which can be designated a-e and g, respectively. See D.L. Anton et al., J. Amer. Chem. Soc., 102, 2215 (1980). The molecule shown in Figure 1 can be abbreviated as shown below:



wherein, e.g., X is CN, OH, CH_3, or adenosyl.

Methylcobalamin serves as the cytoplasmic coenzyme for ^{5}N-methyltetrahydrofolate:homocysteine methyl transferase (methionine synthetase, EC 2.1.1.13), which catalyzes the formation of methionine from homocysteine. Adenosylcobalamin is the mitochondrial coenzyme for methylmalonyl CoA mutase (EC5.4.99.2) which interconverts methylmalonyl CoA and succinyl CoA.

All forms of vitamin B_{12} (adenosyl-, cyano-, hydroxo-, or methylcobalamin) must be bound by the transport proteins, Intrinsic Factor and Transcobalamin II to be biologically active. Specifically, gastrointestinal absorption of vitamin B_{12} relies upon the intrinsic factor-vitamin B_{12} complex being bound by the intrinsic factor receptors in the terminal ileum. Likewise, intravascular transport and subsequent cellular uptake of vitamin B_{12} throughout the body is dependent upon transcobalamin II and the cell membrane transcobalamin II receptors, respectively. After the transcobalamin II-vitamin B_{12} complex has been internalized, the transport protein undergoes lysozymal degradation, which releases vitamin B_{12} into the cytoplasm. All forms of vitamin B_{12} can then be interconverted into adenosyl-, hydroxo-, or methylcobalamin depending upon cellular demand. See, for example, A.E. Finkler et al., Arch. Biochem. Biophys., 120, 79 (1967); C. Hall et al., J. Cell Physiol., 133, 187 (1987); M.E. Rappazzo et al., J. Clin. Invest., 51, 1915 (1972) and R. Soda et al., Blood, 65, 795 (1985).

Cells undergoing rapid proliferation have been shown to have increased uptake of thymidine and methionine. (See, for example, M.E. van Eijkeren et al., Acta Oncologica, 31, 539 (1992); K. Kobota et al., J. Nucl. Med., 32, 2118 (1991) and K. Higashi et al., J. Nucl. Med., 34, 773 (1993)). Since

methylcobalamin is directly involved with methionine synthesis and indirectly involved in the synthesis of thymidylate and DNA, it is not surprising that methylcobalamin as well as Cobalt-57-cyanocobalamin have also been shown to have increased uptake in rapidly dividing tissue (for example, see, B.A. Cooper et al., Nature, 191, 393 (1961); H. Flodh, Acta Radiol. Suppl., 284, 55 (1968); L. Bloomquist et al., Experientia, 25, 294 (1969)). Additionally, upregulation in the number of transcobalamin II receptors has been demonstrated in several malignant cell lines during their accelerated thymidine incorporation and DNA synthesis (see, J. Lindemans et al., Exp. Cell. Res., 184, 449 (1989); T. Amagasaki et al., Blood, 26, 138 (1990) and J.A. Begly et al., J. Cell Physiol., 156, 43 (1993).

Vitamin B_{12} has several characteristics which potentially make it an attractive *in vivo* tumor imaging agent. Vitamin B_{12} is water soluble, has no known toxicity, and in excess is excreted by glomerular filtration. In addition, the uptake of vitamin B_{12} could potentially be manipulated by the administration of nitrous oxide and other pharmacological agents (D. Swanson et al., Pharmaceuticals in Medical Imaging, MacMillan Pub. Co., NY (1990) at pages 621-628).

Bacteria naturally insert Cobalt-59 into the corrin ring of vitamin B_{12}. Commercially this has been exploited by the fermentative production of Co-56, Co-57, Co-58, and Co-60 radiolabeled vitamin B_{12}. For example, see Chaiet et al., Science, 111, 601 (1950). Unfortunately Cobalt-57, with a half life of 270.9 days, makes Co-57-cyanocobalamin unsuitable for clinical tumor imaging. Other metal ions (cobalt, copper and zinc) have been chemically inserted into naturally occurring descobaltocorrinoids produced by *Chromatium* and *Streptomyces olivaceous*. Attempts to chemically insert other metal ions in these cobalt free corrinoid rings has been unsuccessful. The placement of metals (cobalt, nickel, palladium, platinum, rhodium, zinc, and lithium) into a synthetic corrin ring has not presented any major difficulties. However, their instability and cost to produce makes them impractical for biological assays. Although Co-59 is a weakly paramagnetic quadrapolar nuclei in the 2^+ oxidation state,

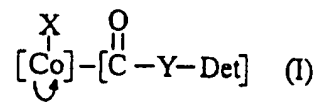
Co-59 exists in the 3^+ oxidation state within the corrin ring of vitamin B_{12} and is diamagnetic. Therefore, insertion of either a radioactive or paramagnetic metal ion other than cobalt into the corrin ring does not seem feasible at this time.

A process for preparing ^{125}I-vitamin B_{12} derivatives is described in Niswender et al. (U.S. Pat. No. 3,981,863). In this process, vitamin B_{12} is first subjected to mild hydrolysis to form a mixture of monocarboxylic acids, which Houts, infra, disclosed to contain mostly the (e)-isomer. The mixture is then reacted with a p-(aminoalkyl)phenol to introduce a phenol group into the B_{12} acids (via reaction with one of the free carboxylic acid groups). The mixed substituent B_{12} derivatives are then iodinated in the phenol-group substituent. This U.S. patent teaches that the mixed ^{125}I-B_{12} derivatives so made are useful in the radioimmunoassay of B_{12}, using antibodies raised against the mixture.

T. M. Houts (U.S. Pat. No. 4,465,775) reported that the components of the radiolabelled mixture of Niswender et al. did not bind with equal affinity to IF. Houts disclosed that radioiodinated derivatives of the pure monocarboxylic (d)-isomer are useful in assays of B_{12} in which IF is used. However, although Houts generally discloses that the monocarboxylic (d)-isomer can be labelled with fluorophores or enzymes and used in competitive assays for vitamin B_{12} in fluids, a continuing need exists for labelled vitamin B_{12} derivatives suitable for tumor and organ imaging and therapy.

Summary of the Invention

The present invention provides detectable compounds of the general formula (I):



(I)

wherein the moiety  is cobalamin, X is CN, OH, methyl or adenosyl, 

is the residue of a monocarboxylic acid of the cobalamin, derived from a corrin propionamide group, and is preferably the essentially pure (b)-, (d)-, or (e)-monocarboxylic acid; Y is a linking group and Det is a chelating group comprising a detectable metal, such as a radionuclide or paramagnetic metal ion. Preferably, the linking group is $-N(H)(CH_2)_{2-6}NH-$.

For example, compounds of formula (I) derived from the (b)-monocarboxylic acid, wherein Det is the diethylenetriaminepentaacetic acid group (DTPA), were prepared comprising Tc-99n, In-111 and Gd-153. These compounds were found to be readily absorbed through the mammalian peritoneal membrane and gastrointestinal tract, to localize within the liver, kidney, pancreas, and spleen. Therefore, the present compounds can be used to evaluate hepatic, splenic, renal, pancreatic, and small bowel function in mammals such as humans and experimental animals, by administering a compound of formula (I) to the mammal and detecting its presence in the target organ, using appropriate normal control values for comparison.

Certain neoplastic tissue has been found to act as a vitamin B_{12} sink, accumulating the vitamin to a greater extent than the surrounding slower dividing tissue. Therefore, the present compounds can also be used for tumor imaging and/or targeted cancer therapy, by administering a compound of formula (I) to a mammal afflicted with a tumor, so that the compound localizes in the tumor, and optionally, detecting the presence of the compound in the tumor, particularly tumors of the organs listed above.

Intermediates useful in the preparation of the compounds of formula (I) are also an aspect of the invention, including compounds wherein Det is replaced by Chel, which is an organic chelating group, or chelator, capable of chelating a radionuclide or radioisotope.

Brief Description of the Figures

Figure 1 depicts the structure of vitamin B_{12}, wherein X is CN (cyano), OH, CH_3 or adenosyl.

Figure 2 schematically depicts the synthesis of a cobalamin metal ion DTPA complex.

Detailed Description of the Invention

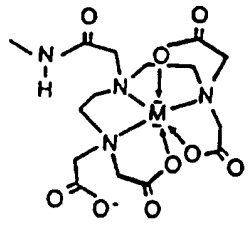
The compounds of formula I can be prepared by producing a monocarboxylic acid of X-[cobalamin], wherein X is cyano-, methyl, adenosyl, and the like. These compounds can be prepared by the mild acid hydrolysis of cyanocobalamin, which has been shown to yield a mixture of mono-, a dicarboxylic acids and one tricarboxylic acid. These carboxylic acids are derived from the propionamide side chains designated b, d and e, as discussed hereinabove, which are more susceptible to hydrolysis than the amide groups on acetamide side chains a, c, and g. The (b)-, (d)-, and (e)-monocarboxylic acids can be separated by column chromatography. See Figure 1 herein, and Figure 1 of D.L. Anton et al., J. Amer. Chem. Soc., 102, 2215 (1980). See, also, J.B. Armitage et al., J. Chem. Soc., 3349 (1953); K. Bernhauer, Biochem. Z., 344, 289 (1966); H.P.C. Hogenkamp et al., Biochemistry, 14, 3707 (1975); and L. Ellenbogen, in "Cobalamin," Biochem. and Pathophysiol., B. Babior, ed., Wiley, N.Y. (1975) at chapter 5.

The X-[cobalamin] [CO_2H] can be linked to the metal chelator by means of a linking group, which is preferably a divalent, or "bifunctional" organic linking group. Such linking groups comprise two reactive groups, one that is coupled to the CO_2H group, and the other that is coupled to the metal chelator. A variety of homobifunctional and heterobifunctional linking reagents known in the art are useful in the present invention. Preferred linkers comprise one or two amino or hydroxyl groups, such as ω-aminoalkanoic acids, e.g., ϵ-amino caproic acid (H_2N-$(CH_2)_5$-COOH), or alkane diamines including 1,4-diaminobutane, 1, 5-diaminopentane and 1,6-diaminohexane, and the like. Particularly preferred among the aminoalkanoic acids and similar compounds are those which are soluble in aqueous buffers.

Det is a chelating group comprising a radionuclide, such as a metallic radioisotope. Preferred among these chelating compounds "chelators"

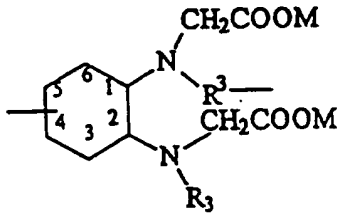
or (chel) are such polycarboxylic acids as EDTA, DTPA, DCTA, DOTA, TETA, or analogs or homologs thereof.

DTPA (diethylenetriaminepentaacetic acid) can be attached to cobalamin carboxylic acid(s) via reaction of diethylenetriaminepentaacetic dianhydride (Aldrich Chem. Co.) with a linker comprising a free amino group. This yields a Chel group that is 2-(amidomethyl)-1,1,7,7-diethylenetriaminetetraacetic acid. This chelator can be reacted with radionuclides to yield a Det moiety of the general formula



wherein M is the radionuclide. The synthetic route to a cobalamin metal ion DTPA complex (4) is schematically shown in Figure 2, wherein WSC = water soluble carbodiimide.

The chelator (chel) DCTA has the general formula:



DCTA is a cyclohexane-based metal chelator, wherein R^3 may by (C_1-C_4)alkyl or CH_2CO_2-, which may be attached to the Y through positions 4 or 5, or through the group R^3 and which carries from 1 to 4 detectable metal or nonmetal cations (M), monovalent cations, or the alkaline earth metals. Thus, with metals of oxidation state +1, each individual cyclohexane-based molecule

may carry up to 4 metal cations (where both R^3 groups are CH_2COOM). As is more likely, with higher oxidation states, the number of metals will decrease to 2 or even 1 per cyclohexane skeleton. This formula is not intended to limit the molecule to any specific stereochemistry. In particular, both amino functionalities may be either cis or trans to each other.

Other macrocyclic carboxylic acid chelators which can be linked to the cobalamin carboxylic acid via bis-amino linking groups include TETA 1,4,8,11-tetraazacyclotetradecane-N,N',N'',N'''-tetraacetic acid; 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid (DOTA); 1,4,8,12-tetraazacyclopentadecane-N,N',N'',N'''-tetraacetic acid (15N4); 1,4,7-triazacyclononane-N,N',N''-triacetic acid (9N3); and 1,5,9-triazacyclododecane-N,N',N''-triacetic acid (12N3). Bifunctional chelators based on macrocyclic ligands in which conjugation is via an activated arm attached to the carbon backbone of the ligand can be employed as described by M. Moi et al., J. Amer. Chem. Soc., 49, 2639 (1989) (2-p-nitrobenzyl-1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid); S.V. Deshpande et al., J. Nucl. Med., 31, 473 (1990); G. Ruser et al., Bioconj. Chem., 1, 345 (1990); C.J. Broan et al., J. C. S. Chem. Comm., 23, 1739 (1990); and C.J. Anderson et al., J. Nucl. Med., 36, 850 (1995) (6-bromoacetamido-benzyl-1,4,8,11-tetraazacyclotetadecane-N,N',N'',N'''-tetraacetic acid (BAT)).

Any metal capable of being detected in a diagnostic procedure *in vivo* or *in vitro* can be employed as M in the Det moieties. Particularly, any radioactive metal ion capable of producing a diagnostic result in a human or animal body or in an *in vitro* diagnostic assay may be used in the practice of the present invention. Suitable ions include the following: Antimony-124, Antimony-125, Arsenic-74, Barium-103, Barium-140, Beryllium-7, Bismuth-206, Bismuth-207, Cadmium-109, Cadmium-115m, Calcium-45, Cerium-139, Cerium-141, Cerium-144, Cesium-137, Chromium-51, Cobalt-56, Cobalt-57, Cobalt-58, Cobalt-60, Cobalt-64, Erbium-169, Europium-152, Gadolinium-153, Gold-195, Gold-199, Hafnium-175, Hafnium-175-181, Indium-111, Iridium-192, Iron-55, Iron-59, Krypton-85, Lead-210, Manganese-54, Mercury-197,

Mercury-203, Molybdenum-99, Neodymium-147, Neptunium-237, Nickel-63, Niobium-95, Osmium-185 + 191, Palladium-103, Platinum-195m, Praseodymium-143, Promethium-147, Protactinium-233, Radium-226, Rhenium-186, Rubidium-86, Ruthenium-103, Ruthenium-106, Scandium-44, Scandium-46, Selenium-75, Silver-110m, Silver-111, Sodium-22, Strontium-85, Strontium-89, Strontium-90, Sulfur-35, Tantalum-182, Technetium-99m, Tellurium-125, Tellurium-132, Thallium-204, Thorium-228, Thorium-232, Thallium-170, Tin-113, Titanium-44, Tungsten-185, Vanadium-48, Vanadium-49, Ytterbium-169, Yttrium-88, Yttrium-90, Yttrium-91, Zinc-65, and Zirconium-95.

The compounds of formula (I) are preferable dissolved or dispersed in a nontoxic liquid vehicle, such as physiological saline or a similar aqueous vehicle, to the desired concentration. A preselected analytical, diagnostic or therapeutic unit dose is then administered to the test animal or human patient, by oral administration or ingestion or by parenteral administration, as by intravenous or intraperitoneal infusion or injection, to attain the desired *in vivo* concentration. Doses useful for imaging or treating human organs or tumors can be derived, from those found to be effective to image or treat organs in humans *in vitro* or in animal models, such as those described hereinbelow, or from dosages of other labelled vitamin B_{12} molecules, previously employed in animal therapy or imaging.

The invention will be further described by reference to the following detailed examples, wherein cyanocobalamin and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide were purchased from Sigma Chem. Co., St. Louis, MO. Adenosine, 1,4-diaminobutane dihydrochloride, diethylenetriamine pentaacetic (DPTA), hexamethylphosphoramide, 1-hydroxybenzotriazole hydrate, iodomethane and thionylchloride were obtained from Aldrich Chem. Co., Milwaukee, WI. Thin layer chromatography (TLC) silica gel and PET-cellulose sheets were purchased from E. M. Science, Gibbstown, NJ. Tc^{99m} and In^{111} were obtained from Mallinckrodt Medical, Inc. and Gd^{153} was obtained

from Amersham. Other inorganic salts and solvents were obtained in the highest purity available.

UV-visible spectra were recorded on a Hewlett-Packard diode array spectrophotometer. DTPA dianhydride and 5'-chloro-5'-deoxyadenosine were synthesized as described by W.C. Eckelman et al., J. Pharm. Sci., 64, 704 (1975) and K. Kikugawa et al., Tetrahedron Lett., 87 (1971), respectively. The monocarboxylic acids of cyanocobalamin, methylcobalamin-b-carboxylic acid and adenosylcobalamin-b-carboxylic acid were prepared and isolated as described by H.P.C. Hogenkamp, Biochemistry, 13, 2736 (1974); D.L. Anton et al., J. Amer. Chem. Soc., 102, 2215 (1980); R.H. Yamada et al., J. Biol. Chem., 247, 6266 (1972) and D. Dolphin, Methods in Enzymology, XVille, 34-52 (1971). Methylcobalamin, adenosylcobalamin and their derivatives are light sensitive, especially in solution, and all reactions and manipulations were carried out in the dark or in dim light.

All images for the *in vivo* studies were obtained on a GE 500 maxicamera using a LEAP collimator with a 20% window about the 140 keV energy peak of technetium, and a medium energy collimator with a 20% window about the 174 keV and 247 keV energy peaks of Indium. A 256x256 matrix with a dedicated pinnacle computer system was used to collect and analyze the data.

Example 1. Cyanocobalamin-b-(4-aminobutyl)amide. A mixture containing cyanocobalamin-b-carboxylic acid (1.0 g, 0.6 mmol), hydroxybenzotriazole (0.81 g, 6 mmol) and 1,4-diaminobutane dihydrochloride (4.8 g, 30 mmol) in 100 ml of water was adjusted to pH 7.8. 1-Ethyl-3-(3'-dimethylaminopropyl)carbodiimide (1.26 g, 6.6 mmol) was then added, the pH was adjusted to 6.4 and the reaction stirred at room temperature for 24 h. TLC on silica gel using *n*-butanol-acetic acid water (5:2:3) showed the reaction to be complete. Cyanocobalamin-b-(4-aminobutyl)amide was extracted into 92% aqueous phenol and the phenol layer was washed several times with equal volumes of water. To the phenol extract were added 3 volumes of diethylether and 1 volume of acetone. The desired cobalamin was removed from the organic

phase by several extractions with water. The combined aqueous layers were extracted three times with diethylether to remove residual phenol, concentrated to approximately 20 ml *in vacuo* and crystallized from aqueous acetone. Yield 955 mg, 92%.

Example 2. Cyanocobalamin-b-(4-aminobutyl)amide DTPA. Cyanocobalamin-b-(4-aminobutyl) amide (500 mg), 0.3 mmol) was dissolved in 30 ml sat. sodium bicarbonate and treated with solid DTPA dianhydride (1.2 g, 3.4 mmol). The progress of the reaction was monitored by TLC on PEI plates using *n*-butanol-acetic acid-water (5:2:3) as the solvent. After 30 min incubation at room temperature a second 1.2 g of the dianhydride was added. After two additional additions of dianhydride with adjustments of the pH to 8.2 the reaction mixture was incubated overnight. Cyanocobalamin-DPTA adduct was then extracted into 92% aqueous phenol and purified as described above. The preparation was evaporated to dryness *in vacuo* and isolated as a glass. Yield 460 mg, 77%. The cyanobalamin-DTPA adduct behaves as a polyanion on paper electrophoresis in 0.1 M sodium phosphate buffer pH 7.1.

Example 3. Methylcobalamin-b-(4-aminobutyl)amide. Methylcobalamin-b-carboxylic acid (1.0 g, 0.6 mmol) was reacted with diaminobutane dihydrochloride as described above for the cyano derivative. The cobalamin was purified by extraction through phenol (see above) and the resulting aqueous solution was concentrated *in vacuo*. This solution was chromatographed on AG1-X2 200-400 mesh in the acetate form (20x 2.5 cm) and the pass through collected. The pass through was concentrated to approximately 20 ml and the desired cobalamin crystallized from aqueous acetone. Yield 920 mg, 88%. Unreacted methylcobalamin-b-carboxyclic acid was eluted with 1 M acetic acid, concentrated and crystallized from aqueous acetone. Yield 60 mg, 6%.

Example 4. Methylcobalamin-b-(4-aminobutyl)amide DTPA.
Methylcobalamin-b-(4-aminobutyl)amide (500 mg, 0.3 mmol) was dissolved in

30 ml saturated sodium bicarbonate and reacted with solid DTPA dianhydride as described above. The methyl cobalamin-DTPA adduct was purified by extraction through phenol, evaporated to dryness *in vacuo* and isolated as a glass. Yield 600 mg, 96%.

Example 5. Adenosylcobalamin-b-(4-aminobutyl)amide. Adenosylcobalamin-b-carboxylic acid (500 mg, 0.3 mmol) was reacted with diaminobutane dihydrochloride (2.4 mg, 15 mmol) as described above. The cobalamin was purified by extraction through phenol (see above). The resulting aqueous solution was concentrated *in vacuo* and applied to AG-50 X2, 200-400 mesh, in the hydrogen form (20 x 25 cm). The column was washed thoroughly with water to remove hydroxybenzotriazole and the desired cobalamin eluted with 1 M ammonium hydroxide. After an additional extraction through phenol, adenosylcobalamin-b-(4-aminobutyl)amide was isolated as a glass. Yield 366 mg, 77%.

Example 6. Adenosylcobalamin-b-(4-aminobutyl)amide DTPA. Adenosylcobalamin-b-(4-aminobutyl)amide (366 mg, 0.23 mmol) was dissolved in 30 ml saturated sodium bicarbonate and treated with solid DTPA dianhydride (1.0 g, 2.8 mmol) as described above. The cobalamin was purified through phenol (see above). The resulting aqueous solution was concentrated and applied to AG-50 X2, 200-400 mesh, in the hydrogen form (6.0 x 2.5 cm), the column was washed with water and the desired cobalamin eluted with 0.1 M ammonium hydroxide. The solution was evaporated to dryness *in vacuo* and adenosylcobalamin-b-(4-aminobutyl)amide DTPA isolated as a glass. Yield 400 mg, 80%.

Example 7. Interaction with Intrinsic Factor and Transcobalamin Proteins. Under dim light, 1000 µg of the non-labeled methyl-, adenosyl-, and cyanocobalamin-b-(4-aminobutyl)amide-DTPA, as well as 1000 µg of cyanocobalamin and DTPA (Sigma, St. Louis, MO 63178), were separately

dissolved in 10 ml of normal saline at room temperature. Each of the five 1000 µg/10 ml samples were stored in sealed, aluminum-wrapped 10 ml vials to prevent exposure to light. No buffers were added to the solutions. The pH of the solutions, measured by a Beckman 40 pH meter (Beckman Instruments, Fullerton, CA): Cyanocobalamin = 5.75, DTPA = 3.78; cyano, methyl and adenosylcobalamin-DTPA analogues were 5.75, 6.10, and 6.19, respectively.

To assess *in vitro* binding to Intrinsic Factor (IF) and Transcobalamins (TC), the intrinsic factor blocking antibody (IFBA) and Unsaturated vitamin B_{12} Binding Capacity (UBBC) assays were performed with serum randomly obtained from five patients being evaluated for pernicious anemia at the Mayo Clinic. The IFBA and UBBC assays were first performed for clinical purposes as previously described by V.F. Fairbanks et al., Mayo Clin. Proc., 58, 203 (1983); Intrinsic Factor Blocking Antibody [^{57}Co] Radioassay-Package insert, Diagnostic Products Corp.; D. Grossowicz et al., Proc. Exp. Biol., 109, 604 (1962) and C. Gottlieb et al., Blood, 25, 6 (1965).

Next, the serum from the same five patients underwent modified IFBA and UBBC assays. Specifically, 1 µl of the five previously described solutions were separately incubated with purified IF or serum, to potentially saturate all IF and TC binding sites. After incubation for 20 minutes at room temperature and for another 20 minutes at 4°C, 500 µl of the stock (1000 µg/l) Cobalt-57-cyanocobalamin (Mallinckrodt Medical, Inc., St. Louis, MO 63134) solution was added and the usual IFBA and UBBC protocols were then followed. All supernatant activity was counted for four minutes on a gamma counter (Micromedix 10/20, Huntsville, AL 35805). The results are shown in Table I.

Table 1

UBBC						
	Clinical Run	CNB_{12}	$MEB_{12}DTPA$	$ADB_{12}DTPA$	$CNB_{12}DTPA$	DTPA
PT1	741	<NSB	17.1	54.6	222.6	731.5
PT2	632	< NSB	26.8	62.6	216.9	913.1
PT3	2097	< NSB	278.9	590.3	713.3	2078.9
PT4	1378	< NSB	60.9	126.9	433.2	1633.7
PT5	1682	< NSB	91.1	163.9	643.2	1418.0

IFBA						
	Clinical Run	CNB_{12}	$MEB_{12}DTPA$	$ADB_{12}DTPA$	$CNB_{12}DTPA$	DTPA
PT1	11942.5 (0.99)	951.5 (12.48)	4279 (2.77)	6758.5 (2.30)	5151 (2.30)	11899 (0.99)
PT2	11656 (1.02)	920.5 (12.90)	4082 (2.92)	6841.5 (1.74)	5133.5 (2.31)	11696.5 (1.02)
PT3	11780 (1.01)	912.5 (13.01)	4456.5 (2.66)	6828.5 (1.74)	5338.5 (2.22)	11735.5 (1.01)
PT4	11617 (1.02)	749 (15.85)	4414 (2.69)	7046.5 (1.64)	6002.5 (1.98)	11909 (1.00)
PT5	11653.5 (1.02)	858.5 (10.91)	4381.5 (2.77)	7096.5 (1.72)	5973.5 (1.99)	11778.5 (1.02)

NSB = Nonspecific binding; counts < 100 consistent with saturation of transcobalamin proteins
Negative reference for IFBA; no binding to intrinsic factor (< 1.11)
Positive reference for IFBA; binding to intrinsic factor (> 1.43)
Indeterminate reference value (1.11 - 1.43)
Clinical Run = patients supernatant counts from UBBC and IFBA assays
DTPA = diethylenetriamine pentaacetic acid
CNB_{12} = cyanocobalamin
$MEB_{12}DTPA$ = methylcobalamin-b-(4-aminobutyl)-amide-DTPA
$ADB_{12}DTPA$ = adenosylcobalamin-b-(4-aminobutyl)-amide-DTPA
$CNB_{12}DTPA$ = cyanocobalamin-b-(4-aminobutyl)-amide-DTPA

The IFBA assay demonstrated that DTPA does not significantly bind to IF (values less than the negative reference), whereas cyanocobalamin and the cobalamin-DTPA analogs do, in varying degrees, competitively inhibit Co-57 cyanocobalamin from binding to intrinsic factor. By using the counts of the Clinical run divided into the counts of the five solutions, the efficacy of binding to intrinsic factor can be estimated. The averaged percent binding of the five solutions to IF was: cyanocobalamin = 92.5%; methylcobalamin-b-(4-aminobutyl)-amide-DTPA = 63.2%; cyanocobalamin-b-(4-aminobutyl)-amide-DTPA = 52.9%; adenosylcobalamin-b-(4-aminobutyl)-amide-DTPA = 41.0% and 0.8% for DTPA. This is in contrast to the disclosure in Houts (U.S. Pat. No. 4,465,775) that the (b)-monocarboxylic acid of vitamin B_{12} and its radioiodinated derivative exhibit very low binding to IF.

Likewise the averaged percent binding of the five solutions to the transcobalamin proteins was: cyanocobalamin = 100%, methylcobalamin-b-(4-aminobutyl)amide-DTPA = 94.0%, adenosylcobalamin-b-(4-aminobutyl)amide-DTPA = 90.4%, cyanocobalamin-b-(4-aminobutyl)amide-DTPA = 66.4% and 3.6% for DTPA.

Thus, the attachment of DTPA to vitamin B_{12} does alter its binding to the carrier proteins. As expected, non-labeled cyanocobalamin had the greatest affinity for IF and the transcobalamin proteins. Methylcobalamin-b-(4-aminobutyl)amide-DTPA was next, followed by adenosylcobalamin-b-(4-aminobutyl)amide-DTPA, and finally cyanocobalamin-b-(4-aminobutyl)amide-DTPA. There was also some nonspecific binding of DTPA to the carrier proteins (0.8% and 3.6%).

Example 8. Chelation of Radionuclides. Under dim light, 1000 µg of methyl-, adenosyl-, and cyanocobalamin-b-(4-aminobutyl)amide-DTPA were separately dissolved in 200 µl of normal saline. Next, 500 µCi of Indium-111 or 250 µCi of Gadolinium-153 were added to the cobalamin-DTPA solutions. The reactions were carried out at room temperature and room air. For the chelation of technetium, the dissolved cobalamin DTPA complexes were separately placed

into sealed 2 ml vials. Next, 200 µl of stannous chloride solution (1000 µg/ml normal saline) were added to each vial. The vials were purged with nitrogen gas for 5 minutes. After this time, 1-5 mCi of Technetium-99m was added to the N_2 purged vials. Each vial underwent further nitrogen purging for 5 minutes. All chelation reactions were mixed gently for 5 minutes.

Control mixtures of 1000 µg of cyanocobalamin were dissolved in 200 µl of normal saline. Cyanocobalamin was mixed with Tc-99m at room temperature and room air, as well as within nitrogen purged vials containing 200 µl of the described stannous chloride solution. Additionally, the cobalamin-DTPA complexes underwent Tc-99m labeling in open vials at room air in the absence of the stannous chloride.

Specific activity was assessed by mixing 100 µl aliquots of methyl and adenosyl cobalamin-b-(4-aminobutyl)amide-DTPA (5 µg/100 µl normal saline) with 50 µl stannous chloride solution (1 µg/50 µl normal saline) in nitrogen purged 2 ml vials. Technetium-99m in 10, 25, 50, 75, and 100 mCi allotments of activity were added to the vials. The vials underwent gentle mixing and continuous nitrogen purging for five minutes after the addition of technetium.

Efficiency of chelation and specific activity were assessed via thin layer chromatography (TLC). Thin layer chromatographic strips (Grade 31 ET Chr-thickness 0.50 mm, flow rate (water) 225 mm/30 min, Whatman Lab Sales, Hilsboro, OR 97123) were developed in acetone in dim light. The dry strips were placed on film (Ektascan-MC1, Eastern Kodak, Rochester, NY 14650) for autoradiography (AR). Chromatographic and autoradiographic results were visually compared. All the radiolabeled cobalamin-DTPA complexes underwent TLC and AR to confirm 100% labeling prior to *in vivo* administration.

Under acetone development, free Tc-99m migrates to the top of the chromatographic strip, whereas In-111 and Gd-153 diffusely spread over the lower two-thirds of the strip. TLC and AR analysis demonstrated that there was 100% labeling of all three cobalamin-DTPA complexes with Tc-99m, In-111,

and Gd-153. Specifically, all radioactivity was confined to the chromatographic distribution of the cobalamin analogues.

Since methyl and adenosyl cobalamin could potentially have greater uptake in malignant tissue, the chelation of Tc-99m, In-111, and Gd-153 by methyl and adenosylcobalamin-b-(4-aminobutyl)amide-DTPA underwent greater scrutiny. The chromatographic and autoradiographic images were consistently coincident. In contrast, unmodified cyanocobalamin did not demonstrate any affinity for binding the three radionuclides. As expected, there was minimal labeling of the cobalamin-DTPA complexes with Tc-99m in the absence of stannous chloride and hypoxic conditions.

At a concentration of 5 μg/100 μl the red color of the cobalamin-DTPA analogues is barely discernible in the aqueous state, and undetectable on TLC. However, the AR distribution is the same when compared to the more concentrated cobalamin analogue solutions with lower specific activity. Methyl and adenosyl cobalamin-b-(4-aminobutyl)amide-DTPA can chelate up to 50 mCi of technetium-99m per 5 μg with 100% efficiency. This results in a specific activity of 10 mCi/μg for the cobalamin-DTPA analogue.

Example 9. In Vivo Studies.

A. Biodistribution: Methylcobalamin-b-(4-aminobutyl)amide-DTPA in a concentration of 300 μg/100 μl normal saline was labeled with 3 mCi of Indium-111. The labeled vitamin B_{12} analogue was diluted with normal saline to a final volume of 1000 μl. Via intraperitoneal injection (IP), five 12 week old female Balb-C mice (Harlan, Sprague, Dawley, Indianapolis, IN 46229) each received 200 μl (500 μCi) of the methylcobalamin-DTPA-^{111}In complex. For comparison, Indium-111-DTPA having the same concentration and specific activity of the methylcobalamin-DTPA analogue, was injected IP into three mice. All mice were sacrificed at 24 hours via CO_2 inhalation. The pancreas, spleen, kidneys, and heart were dissected in their entirety. A portion of the liver, lung, left quadricep muscle, and flank fat were also harvested. All tissue samples and organs were weighed wet, minced in 2.0 ml normal saline, and

counted for five minutes in a gamma well counter (Minaxi Autogamma 5000, Packard Instrument, Downers Grove, IL 60515).

B. Gastrointestinal Absorption: Methylcobalamin-b-(4-aminobutyl)-DTPA and DTPA alone were labeled as described above, with the exception that the 3 mCi Indium/300 μg/100 μl normal saline solutions were not diluted. Two groups of three mice had a few drops of either ^{111}In-DTPA or methylcobalamin-b-(4-aminobutyl)-DTPA-In-111 placed in their oral cavities. The mice were sacrificed at 24 hrs, dissected, and studied as described above.

A modified Schillings test was performed on two mice. Specifically, each mouse received via subcutaneous and intraperitoneal administration, a 1000 μg loading dose of non-labeled methylcobalamin-b-(4-aminobutyl)amide-DTPA analogue. At 24 hrs, the mice were fed 2-3 drops of Indium-labeled methylcobalamin-b-(4-aminobutyl)amide-DTPA-complex. Urine and feces were collected from the three groups of mice after oral administration. The mice were sacrificed at 24 hours after ingestion of tracer and images and biodistribution data were obtained at that time.

C. Tumor Imaging: At 24 hours, there was a significant amount of adenosylcobalamin-b-(4-aminobutyl) amide-DTPA-In-111 uptake within the transplanted sarcoma both visually and by gamma well counting (Table II).

Table II

	Kidney	Liver	Spleen	Pancreas	Heart	Lung	Fat	Muscle	Tumor
Mouse 1	3717.5	943.3	433.1	304.2	134.7	130.9	101.4	93.6	–
Mouse 2	3299.5	823.4	405.3	319.9	189.4	180.1	147.3	51.4	–
Mouse 3	3462.7	768.6	366.8	310.3	171.2	113.1	102.8	43.9	–
Mouse 4	224.0	56.9	44.1	13.4	10.3	6.2	12.6	5.4	–
Mouse 5	130.2	41.5	26.2	13.0	6.9	6.0	19.5	5.6	–
Mouse 6	281.6	66.1	57.7	14.1	12.5	10.5	18.8	5.0	–
Mouse 7	621.4	126.4	67.8	40.0	35.0	38.4	–	13.6	–
Mouse 8	700.5	111.7	66.6	39.3	29.8	51.2	–	12.4	–
Mouse 9	601.7	115.8	66.3	41.2	31.3	40.6	–	12.0	–
Mouse 10	119.4	24.0	19.5	6.0	5.6	5.4	–	8.9	–
Mouse 11	117.3	25.5	19.0	6.7	5.0	5.3	–	2.6	–
Mouse 12	110.1	23.2	18.1	5.9	4.8	5.0	–	3.7	–
Mouse 13	4.3	0.82	0.67	0.75	0.54	1.1	< BKG	< BKG	–
Mouse 14	4.1	0.80	0.70	0.76	0.54	0.33	< BKG	< BKG	–
Mouse 15	3.1	0.73	0.65	1.1	0.50	0.44	< BKG	< BKG	–
Mouse 16	0.64	0.28	0.62	0.93	< BKG	< BKG	< BKG	< BKG	–
Mouse 17	0.54	0.21	0.67	0.96	< BKG	< BKG	< BKG	< BKG	–
Mouse 18	0.59	0.30	0.48	0.61	< BKG	< BKG	< BKG	< BKG	–
Mouse 19	3886.9	691.0	576.3	445.0	165.0	318.8	76.0	70.1	954.7
Mouse 20	3115.6	464.8	309.5	242.7	134.8	230.0	170.4	81.9	1426.0
Mouse 21	3592.8	675.0	478.3	439.0	157.8	335.2	198.0	166.5	1183.1
Mouse 22	116.5	19.7	17.3	7.1	5.0	4.5	13.7	7.2	52.8
Mouse 23	180.7	40.9	22.8	11.3	8.0	9.2	17.9	6.4	69.3
Mouse 24	231.2	60.3	46.1	13.9	9.7	8.5	19.2	6.8	73.1
Mouse 25	543.9	116.5	54.7	38.4	21.7	34.4	39.5	23.5	135.5
Mouse 26	240.8	56.2	25.8	21.3	11.4	19.9	13.5	15.5	60.4
Mouse 27	459.2	107.6	37.1	30.3	16.9	21.3	17.8	14.5	120.3
Mouse 28	14.0	1.6	1.9	1.4	0.94	1.7	0.93	.68	5.0
Mouse 29	9.9	1.3	1.4	8.2	0.61	0.87	0.75	.60	2.8
Mouse 30	10.2	1.4	1.6	3.1	0.85	0.93	0.79	.63	3.4

Mice 1-3 and 19-21 = 500 μCi adenosylcobalamin-b-(4-aminobutyl)-amide-DTPA-^{111}In injected intraperitoneal

Mice 4-6 and 22-24 = 500 μCi DTPA-^{111}In injected intraperitoneal

Mice 7-9 = 500 μCi adenosylcobalamin-b-(4-aminobutyl)-amide-DTPA-^{111}In injected subcutaneously

Mice 10-12 = 500 μCi DTPA-^{111}In injected subcutaneously

Mice 13-15 = approximately 30 μCi methylcobalamin-b-(4-aminobutyl)-amide-DTPA-^{111}In administered orally

Mice 16-18 = approximately 30 μCi DTPA-^{111}In administered orally

Mice 25-27 = approximately 100 μCi methylcobalamin-b-(4-aminobutyl)-amide-DTPA-^{111}In tailvein injection

Mice 28-30 = approximately 100 μCi DTPA-^{111}In tailvein injection

Despite the difference in the amount of activity injected between IP and IV routes, the degree of uptake within the tumor was consistently second behind the kidneys. The tumors had two to four times greater activity than the liver, spleen, and pancreas, with 4-12 times greater activity than that of the heart, lungs, fat, and muscle. As expected, no activity was seen to localize in the left flank of the control mice. Usual uptake in the liver and spleen was again seen. Gross pathology of the dissected masses demonstrated fat encapsulated tumors. Microscopically, by H & E stain, the tumors were solid masses of blue stained cells consistent with a sarcoma. No areas of necrosis were noted.

Although DTPA-^{111}In demonstrated uptake within the transplanted tumors, its concentration was 10-20 times less than that of adenosylcobalamin-DTPA-^{111}In.

D. Intravenous Administration: One milligram of either methyl or adenosylcobalamin-b-(4-aminobutyl)amide-DTPA was labeled with 5 mCi of ^{99m}Tc as described above. Several mice were sacrificed via CO_2 inhalation at varying time intervals after tailvein injection. The first urine passed was collected and analyzed via TLC and AR.

E. Results

1. *In Vivo* Studies

(a) Biodistribution

The organ and tissue distribution of the methyl and adenosylcobalamin-DTPA analogs at 24 hours was similar despite the route of administration (Table II). The kidneys were first, followed by the liver and spleen. The pancreas usually was next followed by the lungs, fat, heart, and muscle. The differences in activity between the pancreas, heart, lung, fat, and muscle was less significant after oral, subcutaneous, and intravenous administration. However, the ratio of uptake between the kidneys to liver, liver to spleen, and spleen to pancreas was relatively constant. The route of administration (IV, IP, PO) did not have any obvious effect on the chelation of Tc-99m or In-111 by these complexes.

The greatest amount of DTPA-^{111}In uptake was in the kidneys. The distribution of DTPA was similar to the cobalamin analogs, especially after intraperitoneal injection. Despite their similarities, DTPA-^{111}In had 5-12 times less activity per organ or tissue sample when compared to the methyl and adenosylcobalamin analogs.

(b) Gastrointestinal Absorption

Methylcobalamin-b-(4-aminobutyl) amide-DTPA-In-111 was absorbed from the gastrointestinal tract after oral administration. The majority of activity was localized in the kidneys, liver, and spleen on delayed imaging. In the mice that were not "flushed" with oral and intraperitoneal doses of non-labeled methylcobalamin-b-(4-aminobutyl) amide-DTPA, no discernable activity was detected in the urine by gamma well counting. However, the mice that underwent the "modified Schillings test" had detectable radioactivity within their urine at one hour. Imaging at 24 hours of these "flushed" mice demonstrated significantly less activity throughout the body when compared to the "non-flushed" mice. Fecal radioactivity became detectable at 2 hours in both groups receiving the radioactive cobalamin analogs orally.

DTPA-^{111}In was also absorbed from the gastrointestinal tract, but to a lesser degree. No activity was detected in the heart, lungs, muscle, or fat tissue samples. Radioactivity was detected in urine and stool by two hours.

(c) Intravenous Administration

Micturition occurred at approximately 15 and 45 minutes after intravenous and intraperitoneal injections, respectively. The first passed urine after intravenous or intraperitoneal administration was always radioactive. TLC and AR analysis of the collected urine showed no evidence of dissociation of the Tc-99m or In-111 from the cobalamin-DTPA complexes. Images at 5 minutes and 4 hours after tailvein injection demonstrated focal early uptake in the kidneys which became obscured by the liver and spleen activity on the delayed images.

(d) Tumor Imaging

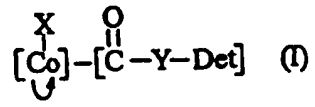
At 24 hours, there was a significant amount of adenosylcobalamin-b-(4-aminobutyl) amide-DTPA-In-111 uptake within the transplanted sarcoma both visually and by gamma well counting (Table II). Despite the difference in the amount of activity injected between IP and IV routes, the degree of uptake within the tumor was consistently second behind the kidneys. The tumors had two to four times greater activity than the liver, spleen, and pancreas, with 4-12 times greater activity than that of the heart, lungs, fat, and muscle. As expected, no activity was seen to localize in the left flank of the control mice. Usual uptake in the liver and spleen was again seen. Gross pathology of the dissected masses demonstrated fat encapsulated tumors. Microscopically, by H & E stain, the tumors were solid masses of blue stained cells consistent with a sarcoma. No areas of necrosis were noted.

Although DTPA-^{111}In demonstrated uptake within the transplanted tumors, its concentration was 10-20 times less than that of adenosylcobalamin-DTPA-^{111}In.

The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the scope of the invention.

WHAT IS CLAIMED IS:

1. A compound of the formula:



(I)

wherein the moiety  is cobalamin,  is the residue of a monocarboxylic acid of cobalamin, X is CN, OH, methyl or adenosyl, Y is a linking group and Det is a detectable chelating group comprising a radionuclide or a paramagnetic metal ion.

2. The compound of claim 1 wherein the radionuclide is a metallic radioisotope.

3. The compound of claim 2 wherein the metallic radioisotope is Tc^{99m}, In^{111} or Gd^{153}.

4. The compound of claim 1 wherein  is the residue of the (b)-monocarboxylic acid.

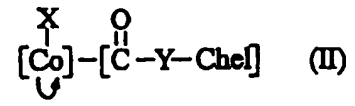
5. The compound of claim 4 wherein Y is a divalent monomer, dimer or trimer of $N(H)(CH_2)_{2-6}N(H)$.

6. The compound of claim 5 wherein Y is $-N(H)(CH_2)_4NH-$.

7. The compound of claim 1 wherein Det is EDTA, DTPA, DOTA, TETA, or DCTA.

8. The compound of claim 3 wherein Det comprises DTPA.

9. A compound of the formula:



(II)

wherein the moiety  is cobalamin,  is the residue of a monocarboxylic acid of the cobalamin, X is CN, OH, methyl or adenosyl, Y is a linking group and Chel is a chelating group which can chelate a radionuclide or a paramagnetic metal ion.

10. The compound of claim 9 wherein Chel is EDTA, DTPA, DOTA, TETA, or DCTA.

11. The compound of claim 9 wherein Y is a divalent monomer, dimer, or trimer of $-N(H)(CH_2)_{2-6}N(H)-$.

12. The compound of claim 11 wherein Y is $-N(H)(CH_2)_4NH-$.

13. The compound of claim 9 wherein  is the residue of the (b)-monocarboxylic acid.

14. A method of evaluating kidney, liver, spleen or intestinal function in a mammal comprising administering to said mammal a detectable amount of a compound of claim 1 in combination with a pharmaceutically acceptable vehicle, and detecting the presence of said compound in the kidney, liver, pancreas, spleen, or intestine of said mammal.

15. The method of claim 14 wherein the administration is parenteral.

16. The method of claim 15 wherein the administration is intravenous.

17. The method of claim 16 wherein the administration is intraperitoneal.

18. The method of claim 14 wherein the administration is oral.

19. A method of detecting a tumor in a mammal afflicted with a tumor comprising administering to said mammal an amount of a compound of claim 1 in combination with a pharmaceutically acceptable vehicle, and detecting the presence of said compound in the tumor.

20. The method of claim 19 wherein the administration is parenteral.

21. The method of claim 19 wherein the administration is oral.

22. The method of claim 19 wherein the vehicle is an aqueous vehicle.

23. The method of claim 19 wherein the tumor is a liver, kidney, splenic, pancreatic, or gastrointestinal tumor.

Figure 1

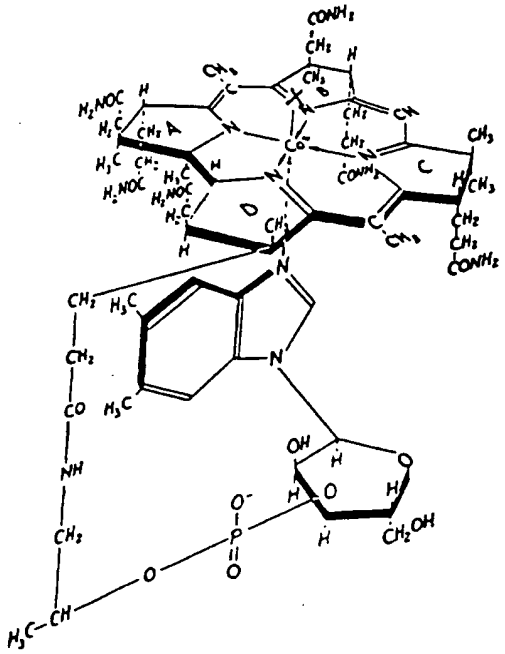
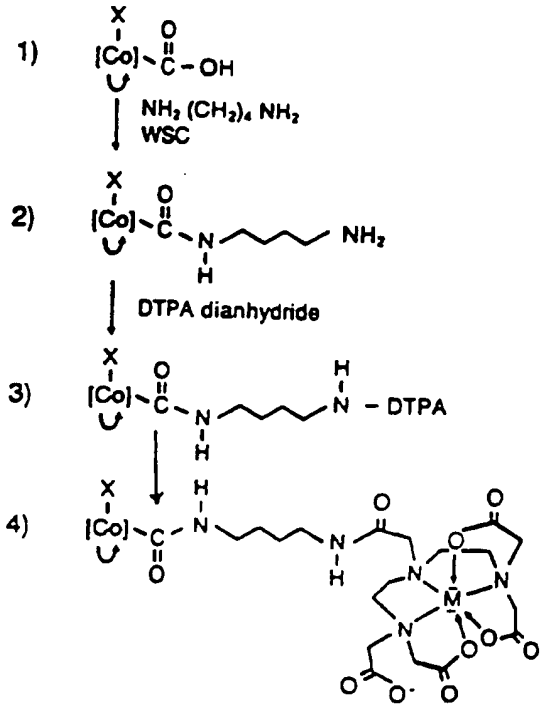


Figure 2



INTERNATIONAL SEARCH REPORT

Interr ~onal Application No
PC.,US 96/18334

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07H23/00 A61K51/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07H A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 165 716 A (MICROMEDIC SYSTEMS, INC.) 27 December 1985 see the whole document ---	1
A	EP 0 069 450 A (TECHNICON INSTRUMENTS CORPORATION) 12 January 1983 see the whole document & US 4 465 775 A (HOUTS, T.M.) 14 August 1984 cited in the application ---	1
A	US 4 283 342 A (YOLLES, S.) 11 August 1981 see the whole document ---	1
A	EP 0 005 834 A (E.R. SQUIBB & SONS) 12 December 1979 see the whole document ---	1
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
26 February 1997	- 5 -03- 1997
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer Rinkel, L

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PC./US 96/18334

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 3 936 440 A (NATH, A.) 3 February 1976 see the whole document -----	1

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 96/ 18334

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 14-23 are directed to a method of treatment of (diagnostic method practised on) the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PC./US 96/18334

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 165716 A	27-12-85	US 4672028 A	09-06-87
		AU 582970 B	13-04-89
		AU 4279885 A	28-11-85
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		AT 295 T	15-10-81
		AU 4649179 A	06-12-79
		CA 1109464 A	22-09-81
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US 3936440 A	03-02-76	NONE	